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(54) Title: PRODUCTION OF BLUETONGUE VIRUS NON-STRUCTURAL PROTEINS USING A BACULOVIRUS EXPRESSION VECTOR

(57) Abstract

Bluetongue virus non-structural proteins (particularly Bluetongue NSI protein) are expressed in insect cells.

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PRODUCTION OF BLUETONGUE VIRUS NON-STRUCTURAL PROTEINS USING A BACULOVIRUS EXPRESSION VECTOR

This invention relates to a process for producing bluetongue virus proteins.

Bluetongue virus (BTV) is the prototype virus of the *Orbivirus* genus (*Reoviridae* family). It is vectored to vertebrates by *Culicoides* species and causes disease in certain ruminants, notably sheep.

The genome of BTV consists of 10 unique double-stranded (ds) RNA molecules, each believed to code for a single polypeptide product (Gorman *et al.*, 1981; Sanger and Mertens, 1983). The ten dsRNA species are contained in an inner core structure that contains five types of proteins, two that are major (VP3 and VP7) and three that are minor components (VP1, VP4 and VP6). The core is surrounded by an outer capsid consisting of two major proteins, VP2 and VP5, to give a complete virion particle with a diameter of approximately 69 nm. In a prior patent application (EP-88301348.4-2105) there is described the production of these BTV structural proteins, particularly VP2 and VP3, in insect cells using a baculovirus expression vector. VP2 is able to elicit neutralizing antibody and VP3 can elicit group-reactive antibody and therefore these antigens may be used for diagnosis of, and as a vaccine against, BTV infection.

In addition to the above-mentioned structural proteins, three non-structural proteins (NS1, NS2 and NS3) appear in BTV infected cells. Their function in the replication or morphogenesis of BTV is not known. Two viral specific entities, tubules and granular inclusion bodies, are routinely obserbed in BTV infected cells (Lecatsas, 1968). These morphological structures are attached to the intermediate filament component of the cell's cytoskeleton (Eaton, B.T. *et al.*, 1988) and are presumed to be involved in the virus assembly process. It has been shown (Huismans and Els, 1979) that the tubular structures are composed entirely of one type of polypeptide, namely the 64,000 Dalton (64 kD) NS1 protein, which is the gene product of BTV dsRNA middle size segment No. 6 (M6).

There is a need to be able to produce additional antigenically active bluetongue virus proteins by recombinant DNA technology so that antigens are available for the effective diagnosis of BTV infection and for the development of vaccines.

Following the successful expression of antigenically competant BTV structural proteins in insect cells outlined above, it has now surprisingly been found that expression systems based on insect cells can efficiently be used to achieve expression of BTV non-structural proteins in antigenic form. Thus according to the present invention there is provided a process for producing a polypeptide comprising a bluetongue virus non-structural protein in antigenic form which comprises infecting susceptible insects or cultured insect cells with an expression vector having a DNA segment coding for said polypeptide. An example of such a polypeptide is the NS1 protein of bluetongue virus.

Surprisingly it has been found that insects and cultured insect cells are capable of producing bluetongue virus non-structural proteins in morphological forms which resemble structures observed in bluetongue-virus infected mammalian cells. Thus, for example, BTV-NS1, when produced in accordance with the invention in insect cells has been found to "self-assemble" into tubules which are similar to tubules observed in bluetongue virus infected sheep cells.

Especially suitable expression vectors are those based on baculoviruses. Thus for example the expression vectors used in the method of the invention may comprise a recombinant baculovirus having a DNA segment coding for a polypeptide comprising a bluetongue virus non-structural protein.

Such recombinant baculoviruses may include promoter systems native to naturally occurring baculoviruses, for example the so-called "polyhedrin" promoter, or they may include other promoter systems capable of directing expression of polypeptide in transformed insect or cultured insect cells.

Especially suitable cultured insect cells are those of *Spodoptera frugiperda*.

By the term "antigenic form" as used herein to refer to a protein is meant a protein which is capable of exhibiting an

antigenic property of a native bluetongue virus protein, e.g. the capability of binding to an antibody to said protein.

The expression and characterisation of the BTV serotype 10 (BTV-10) NS1 gene product using an expression system based on recombinant baculoviruses is illustrated by the following Example. The expressed protein has been shown to be similar in size and antigenic properties to the authentic BTV NS1 protein. It reacts with BTV antibody and forms numerous tubular structures in the cytoplasm of the infected insect cells. The tubular structures have been purified to homogeneity from infected cell extracts. Further the expressed polypeptide has been used to identify antibodies to five United States BTV serotypes in infected sheep sera, indicating the potential of the expressed protein as a group reactive antigen in the diagnosis of BTV infections.

In the Example the BTV-10 M6 gene product is expressed in an insect baculovirus expression vector derived from AcNV.

Example - Expression of BTV NS1

A. Viruses and cells. United States prototype BTV-10 was plaque cloned using monolayers of BHK-21 cells. The viral dsRNA was purified as described by Yamaguchi *et al.* (1988) and the 10 individual RNA segments separated and isolated as described previously (Purdy *et al.*, 1984). AcNPV and recombinant baculovirus stocks were grown and assayed in confluent monolayers of *S.frugiperda* cells in medium containing 10% fetal calf serum according to the procedures described by Brown and Faulkner (1977). Occasionally virus stocks were made using spinner cultures of these insect cells.

B. DNA cloning of the BTV10-M6 RNA. Polyadenylation of BTV-10 dsRNA and synthesis of cDNA copies of the polyadenylated M6 RNA using an oligo$(dT)_{12-18}$ primer were undertaken as described previously (Purdy *et al.*, 1984). The RNA templates were removed by treatment with 0.5 M KOH and double-stranded DNA generated by self-annealing. The products were repaired using the Klenow large fragment of DNA polymerase, followed by 3' tailing with dC and annealing to *Pst*I-cut, dG-tailed, pBR322 plasmid DNA (Maniatis

et al., 1982). After transformation, clones containing the viral sequences were recovered and screened by colony hybridization (Grunstein and Hogness, 1975). *Hinf*I restriction patterns of recombinant plasmids were compared as described previously (Purdy *et al.*, 1984).

C. <u>RNA gel electrophoresis, blotting and hybridization</u>. Purified BTV-10 RNA was resolved on an agarose gel, blotted onto a Genescreen membrane (New England Nuclear, Boston MA) and hybridized to nick-translated cloned DNA by procedures described previously (Purdy *et al.*, 1984).

D. <u>Sequencing of BTV M6 DNA clones</u>. Sequencing was carried out end-labeled, strand-separated, restriction fragments of plasmid DNA containing the viral M6 DNA using the Maxam and Gilbert method (Maxam and Gilbert, 1980).

E. <u>DNA manipulations and construction of a complete BTV M6 gene</u>. Plasmid DNA manipulations were carried out following the procedures described by Maniatis and associates (Maniatis *et al.*, 1982). Restriction enzymes, T4 DNA ligase and the Klenow large fragment of DNA polymerase, were purchased from New England Biolabs, Inc. (Beverly, MA). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim (FGR).

Two M6 DNA clones representing nucleotides 1-1100 (#14) and 700-1769 (#39) were sequenced as reported previously (15, see Fig. 1). The construction of plasmid pBTV 10-6 representing the complete M6 DNA is illustrated in Fig. 2. Clone #14 was initially recloned in the opposite orientation into the P*st*I site of pBR322. The derived clone (#14-9) was then digested with P*vu*I and a fragment of DNA containing M6 residues 1-898 was recovered and dephosphorylated (Fig. 2). A P*uv*I derived fragment was obtained from clone #39 containing M6 residues 899-1769. The two fragments were ligated together. After transformation of *Escherichia coli* MC1061 cells and the selection of drug-resistant colonies followed by colony

hybridization screening (Grunstein and Hogness, 1975) using the nick-translated products of M6 DNA derived from clones #14 and #39, clone pBTV 10-6 was identified and confirmed by the appropriate restriction enzyme and sequence analyses to contain BTV-10 M6 residues 1-1769.

F. <u>Construction of a recombinant baculovirus transfer vector</u>. A 1746 base-pair DNA fragment containing the entire coding region of the BTV-10 M6 DNA, was excised from plasmid pBTV 10-6 with *Mae*I, repaired with the Klenow large fragment of DNA polymerase, then cloned into the *Bam*HI site of the baculovirus transfer vector pAcYM1 (Matsuura *et al.*, 1987). The derived recombinant transfer vector (designated pAcBTV 10-6, Fig. 2) was characterised by restriction enzyme and sequence analyses (Maxam and Gilbert, 1980) and was shown to have the viral insert in the correct orientation for expression directed by the AcNPV polyhedrin promoter.

G. <u>Transfection and selection of recombinant viruses</u>. To obtain recombinant viruses that would express the BTV M6 gene, *S. frugiperda* cells were transfected with mixtures of infectious AcNPV DNA and DNA obtained from plasmid pAcBTV 10-6. Recombinant viruses were obtained as described previously (Inumaru and Roy, 1987). One of the derived recombinant viruses was designated AcBTV 10-6.

H. <u>Analysis of infected cell polypeptides by polyacrylamide gel electrophoresis</u>. *S. frugiperda* cells in 35 mm tissue culture dishes were infected with viruses at a multiplicity of 10 PFU/cell and the cells incubated at 28°C for 3 days. At the end of the incubation period, cells were rinsed three times with phosphate buffered saline (PBS) and resuspended in 100 µl of 10 mM Tris-HCl buffer (pH 7.4). 50 µl of protein dissociation buffer (2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, 0.01% bromophenol blue, pH 6.8) were added to each sample and the mixture heated at 100°C for 10 min. Proteins were analysed by electrophoresis in a 10-30% linear gradient polyacrylamide gel in

the presence of SDS (SDS-PAGE) as described by Laemmli (1970). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue.

I. Immunoblotting analyses. After SDS-PAGE, cellular proteins were transferred electrophoretically for 3 hr at 0.8 mA/cm^2 (Kyhse-Anderson, 1984) into a Durapore membrane (Millipore Corp.) using a semi-dry electroblotter (Sartoblot II Sartorius Corp). After transfer, the membrane was soaked overnight at 4xC in blocking buffer (5% skimmed milk, 0.05% Tween 20 in PBS, pH 7.4). The membrane was then treated for 2 hr with rabbit anti-BTV-10 serum diluted 1000-fold in blocking buffer. Following a second wash with 0.05% Tween-20 in PBS (PBST), the membrane was soaked for 1 hr at room temperature in anti-rabbit IgG-goat IgG-alkaline phosphatase conjugate (Sigma Chemical Co.), also diluted in blocking buffer. After further washing with PBST, bound antibodies were detected by incubating using Fast BB salt and a-Naphthyl phosphate (Sigma Chemical Co.) as substrate.

J. Electron micrographs of infected cells. *S. frugiperda* cells were infected with recombinant baculoviruses at a multiplicity of 10 PFU/cell and incubated at 28xC for 3 days. The cells were washed with PBS, fixed with glutaraldehyde and treated with osmic acid. Cell sections were observed in a JEOL electron microscope.

K. ELISA for the detection of antibody to BTV NS1 polypeptide using recombinant baculovirus derived antigens. A solid phase indirect micro-ELISA was used to demonstrate the reactivity of recombinant AcBTV 10-6 antigen with various polyclonal BTV antisera. *S. frugiperda* cells infected 72 hr previously with recombinant AcBTV 10-6 virus (see above) were collected and subjected to freezing and thawing followed by low speed centrifugation to remove cellular debris. The supernatant was diluted (1:10- to 1:10,000-fold) with sodium carbonate buffer (15 mM Na_2CO_3, 36 mM $NaHCO_3$, pH 9.6). A 96-well polyvinylchloride microplate (Flow Laboratories) was coated overnight at 4xC with 50fl of the diluted antigen. The plate was washed three times between each step of the following protocol by flooding the wells of the plate with PBST

buffer. The antigen-coated microplate was washed and blocked with blocking solution for 3 hr at room temperature. BTV antisera were diluted (1:100- to 1:12,800-fold) in blocking solution and 50µl of diluted sera were added to each well. After 2 hr of incubation the plate was washed and 100 µl of a 1:1000 dilution of anti-sheep IgG-alkaline phosphatase conjugate (Sigma Chemicals) was added to each well followed by 2 hr incubation at 28°C. The substrate, p-nitrophenylphosphate (Sigma Chemicals) solution (0.1% w/v final concentration), was added for 30 min, or less. After suitable colour development at room temperature the reaction was stopped by addition of 0.3 M NaOH. The optical density was read using a multichannel spectrophotometer (Microelisa Autoreader, MR 580, Dynatech Co.) at a wavelength of 405 nm.

L. Purification of tubular stuctures by gradient centrifugation. Cells were infected with the AcBTV 10-6 recombinant baculovirus and the infected cells harvested 4 or 5 days post-infection. The cells were recovered, washed twice with PBS and resuspended in 10 mM Tris-HCl (pH 7.4) and disrupted by sonication. The resulting cell extract was loaded on a 10% to 50% (wt/v) sucrose gradient in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) and centrifuged at 40,000 rpm for 3 hr using an SW41 rotor. After centrifugation, the gradient was fractionated and a portion of each fraction was subjected to gel electrophoresis. The peak fractions containing NS1 protein were pooled and pelleted by centrifugation for 2 hr at 40,000 rpm. The pellet was resuspended in 10 mM Tris HCl buffer (pH 7.4).

The results achieved in the above Example is apparent from the following discussion.

1. Nucleotide sequence determination of the BTV-10 M6 DNA and its predicted gene product. The sequence of the cloned M6 gene was determined on strand separated, end-labeled, restriction DNA fragments obtained from two overlapping clones (#14 and #39) using the strategy shown in Fig. 1. The distance each DNA strand was sequenced is indicated by an arrow. The complete nucleotide sequence of these DNA clones has been reported elsewhere (Lee and Roy, 1987).

Approximately 90% of the gene was sequenced in both directions. Excluding the homopolymeric tails, the gene is 1769 nucleotides long with a single open reading frame beginning with an RNA AUG triplet at residues 35-37. This open reading frame terminates with a UAG translation termination codon at residues #1691-1693 (Lee and Roy, 1987). Based on the sequence data, the BTV-10 M6 RNA codes for a primary gene product composed of 552 amino acids with an estimated size of 64,445 Daltons (Table 1) and an overall net positive charge of +2 at neutral pH. The protein has 16 cysteine residues (3%). The hydropathic profile of the protein (Fig. 3) indicates that it contains several regions of hydrophobic amino acids (regions above the central line) throughout the molecule, particularly in the carboxy terminal half.

2. Construction of baculovirus recombinants. A plasmid (pBTV10-6) containing the complete BTV-10 M6 sequence was constructed from two overlapping clones as described above (Fig. 2). To remove unnecessary sequences (such as the homopolymeric tails, the restriction enzyme *Mae*I was used to isolate the entire coding region of the M6 DNA (including 19 bases upstream from the ATG initiation codon and 59 bases downstream from the TAG stop codon) and the DNA used to prepare a recombinant baculovirus transfer vector.

Previous studies have demonstrated that for many genes the highest baculovirus expression levels are obtained with the transfer vector pAcYM1 (Emery and Bishop, 1987; Matsuura *et al.*, 1987; Schmaljohn *et al.*, 1988). This vector contains the entire upstream sequence of the AcNPV polyhedrin gene, including the A of the initiating ATG codon. The coding sequences of the BTV-10 M6 DNA were therefore inserted into the transfer vector pAcYM1 as described above (Fig. 2). The derived recombinant (pAcBTV 10-6) was analysed by restriction endonuclease digestion and the junction sequences determined (Fig. 3). *S. frugiperda* cells were transfected with mixtures of AcNPV DNA and the plasmid DNA pAcBTV 10-6. Recombinant viruses (e.g., AcBTV 10-6) were identified by their polyhedrin-negative plaque phenotypes and plaque purified three times using monolayers of *S. frugiperda* cells.

3. Expression of bluetongue virus NS1 protein. In order to demonstrate that BTV-10 NS1 protein was synthesized in recombinant baculovirus infected cells, protein extracts were prepared from insect cells infected with the recombinant virus AcBTV 10-6 as described above. Extracts were also made from AcNPV- and mock-infected cells. A sample of each preparation was subjected to SDS-PAGE and the resolved proteins stained with Coomassie brilliant blue. As shown in Fig. 4A (lane c), the recombinant virus synthesized a protein with a molecular size of *ca* 60 kD, in agreement with the estimated size of the BTV-10 NS1 protein (see Table 1). Based on comparisons of stained gels containing known quantities of bovine serum albumin, the amount of this protein was estimated to be of the order of 1 mg per 2 x 10^6 infected cells, i.e., as high as the polyhedrin protein made in AcNPV infected cells (compare Fig. 4A, lanes b and c).

In order to confirm that the 60 kD protein was NS1, a sample of the AcBTV 10-6 infected cell extract was electrophoresed, transferred onto a Durapore membrane and subjected to Western analyses using anti-BTV-10 sera as described in Methods. As shown in Fig. 4B, the 60 kD protein (lane c) was identified by the alkaline phosphatase conjugate detection procedure.

4. Electron microscope analysis of cells infected with recombinants that express the BTV NS1 protein. It has been postulated that the NS1 protein of BTV is the sole component of the tubular structures identified in BTV-infected cells (Huismans and Els, 1979). An examination was therefore undertaken of electron micrographs of *S. frugiperda* cells infected with the AcBTV 10-6 recombinant virus, or with wild-type AcNPV. As shown in Fig. 5b and in contrast to AcNPV infected cells (Fig. 5a), numerous tubular structures were evident only in the cytoplasm in the recombinant virus infected cells (Fig. 5, T arrowheads). In the thin section tubules of various lengths were evident. In cross-section, they exhibited a diameter of *ca* 60 nm. Many of the tubules appeared to contain ribosome-like particles (Fig. 5, R arrowheads). Both the structures and arrangement of the tubules were comparable to the tubular structures reported by others in BTV infected BHK-21 cells

(Huismans and Els, 1979). As expected, polyhedra were only evident in the AcNPV infected cells (Fig. 5, P arrowheads).

Fibrous structures were observed both in AcNPV and in the recombinant virus infected cells (Fig. 5). In the latter, the tubules appeared to be aligned along the edges of bundles of the fibrous material (Fig. 5c and d). Both the tubules and the fiberous structures were randomly oriented with respect to one another. In some cells bundles of fibers were seen in both the nucleus and the cytoplasm (Fig. 5e, f), with tubules mostly, but not exclusively associated with the cytoplasmic fibers. Fewer tubules were seen in the nuclei (Fig. 5f). Whether these tubules were formed in the nucleus, or occurred there because the cell was in the terminal stage of infection, is not known.

In order to assess the possible three-dimensional arrangement of the tubules in the infected *S. frugiperda* cells, single arrays in thin sections were examined in a Jeol electron microscope equipped with a tilting stage. A series of photographs was taken at 10° intervals of specimen tilt. It was apparent from Fig. 6A that the circular particles visualised in the lower left of the picture taken at a -40° tilt transformed to oblique particules at a -30° tilt and were progressively observed as longitudinal tubules at -10° to the +50° tilting stages. Similar transformations were observed for other arrays of tubules in the section (see Fig. 6A). A schematic three-dimensional interpretation of the structures is provided in Fig. 6B.

5. <u>ELISA assays using recombinant virus derived NS1 to identify bluetongue antibodies</u>. Since the NS1 gene (M6) of BTV-10 has been shown to be highly conserved among 20 BTV serotypes (Huismans and Cloete, 1987; Ritter and Roy, 1988), the question of whether the NS1 protein produced by the recombinant baculovirus reacted with homologous and heterologous BTV antisera was investigated using an ELISA test. Recombinant virus infected cell extracts were absorbed to microtiter plates (representing some 50 ng of NS1 protein per well) and incubated with either polyclonal BTV-2, or BTV-10, or BTV-11, or BTV-13, or BTV-17 sheep antisera, or with normal sheep serum as a control. The derived antigen-antibody complexes were

detected by incubating with anti-sheep-alkaline phosphatase conjugates followed by the addition of an enzyme substrate. As shown in Fig. 7, all five BTV antisera reacted with the recombinant antigen in proportion to the end point titer of the antisera. No reaction was detected with the normal sheep serum. No reactivity was obtained when each of the BTV antisera was tested with AcNPVinfected cell extracts.

6. Purified tubules recovered from recombinant virus infected insect cells. In order to purify the tubular structures, infected cell extracts were prepared and subjected to sucrose gradient centrifugation. A portion of each fraction was analysed by SDS-PAGE and peak fractions containing NS1 protein were pooled and pelleted. As shown in Fig. 8, the recovered protein, stained with Coomassie brilliant blue, was effectively purified by this one-step procedure. Electron micrographs revealed that the preparation was composed of tubules with little evidence of cytoplasmic or other contamination.

SUMMARY

Two overlapping cDNA clones representing segment M6 of BTV-10 have been used to determine the complete sequence of the viral RNA. The sequence was deduced to be 1,769 base-pairs long, in good agreement with the 1.8 Kb length calculated by agarose gel electrophoresis. The single open reading frame of significant length is initiated with an AUG at residues 35-37 and codes for a protein of some 552 amino acids. The predicted NS1 protein coded by the M6 RNA has a size of 64,445 Da. slightly larger than the size estimated by Huismans (1979), or estimated the electrophoretic mobility of the protein observed in gels by comparison with protein markers (Fig. 4). Computer analyses of the predicted amino acid sequences of the protein indicated that the molecule has domains of hydrophobic amino acids, particularly near the carboxy terminus, and contains many cysteine residues.

NS1 is the major viral protein synthesized in BTV-infected cells and is a major constituent of the tubules which are observed in such cells. (Huismans and Els, 1979). It has now unexpectedly been found that baculovirus expressed NS1 protein also form tubules

in insect cells. The two overlapping clones (#14 and 39) were used to construct a plasmid containing the complete gene (pBTV 10-6). A recombinant baculovirus was prepared with the NS1 gene sequences under the control of the AcNPV polyhedrin promoter. The data herein shows that the BTV NS1 protein is expressed to a high level in insect cells infected with this recombinant baculovirus. From stained preparation of cell extracts it was estimated that the amount of NS1 present in cells infected at high multiplicity with the recombinant virus was *ca* 50% of the total stainable protein in the cell extract prepared at the end of the infection course. It has further been demonstrated that the recombinant baculovirus makes tubular structures in insect cells similar to those reported in BTV infected cell cultures (Huismans and Els, 1979). The tubules have been purified and demonstrated to be composed of NS1 protein.

Previously it has been reported that the DNA probe representing the BTV M6 RNA hybridized with the corresponding RNA segments of 20 out of 20 BTV serotypes tested, indicating that the gene (and gene product) is highly conserved (Ritter and Roy, 1988).

Moreover, unlike similar data obtained with genes representing the viral structural proteins, no hybridization was obtained with the corresponding segments of two epizootic haemorrhagic disease virus serotypes indicating that NS1 genes are perhaps conserved only by BTV serotypes. If correct, then NS1 antigen would be valuable as a reagent for the diagnosis of bluetongue virus infections. In order to investigate the value of the expressed NS1 protein as a diagnostic reagent, recombinant virus derived NS1 protein was applied in an ELISA test with five available US BTV antisera. The data obtained indicates that the genetically engineered NS1 antigen is suitable for identifying all the US BTV serotypes.

The genes for NS1, NS2 and NS3 can be used as group specific probes to detect the presence of bluetongue virus (all 24 serotypes); Ritter and Roy, 1988.

BTV 10 NS1 antigen can be used to detect antibodies to the US strains (BTV-2, BTV-10, BTV-11, BTV-14 and BTV-17); Urakawa and Roy, 1988.

DEPOSITS

A culture of AcNPV-BTV-10 (NS1) has been deposited at the European Collection of Animal Cell Cultures (ECACC) on 10th August 1989 under Accession No. 89081017.

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FIGURE LEGENDS

Fig. 1 Strategy used to determine the sequences of cDNA clones of the BTV-10 M6 gene. The distance and directions in which individual strands of two overlapping clones were sequenced are shown by the solid arrows. Restriction enzyme sites used are as follows: Ma, *Mae*I; Hd, *Hind*III; H, *Hinf*I; D, DdeI; Pv, *Pvu*I; Ms, *Msp*I.

Fig. 2 Schematic diagram of the construction of the transfer vector pAcBTV10-6. A pBR332-based plasmid (pBTV 10-6) containing the entire coding region of the BTV-10 M6 DNA was constructed from two overlapping partial clones as described in Methods. The complete clone (pBTV10-6) was used to construct the transfer vector (pAcBTV10-6) as described in Methods. The sequence of the 5' insertion site was determined by the method of Maxam and Gilbert (18) using a *Hind*III restriction fragment (BTV DNA residue #296).

Fig. 3 Hydropathic plot and distribution of cysteine residues for the predicted M6 gene product of BTV-10. The regions of the protein that have a net hydrophobicity (areas above the center line), or hydrophilicity (areas below the center line), as well as the distribution of cysteine residues (vertical bars), are displayed (11). The plot involves a span setting of 21 amino acids.

Fig. 4 Expression of NS1 protein by recombinant baculoviruses derived from the pAcBTV10-6 transfer vector. *S. frugiperda* cells were infected with recombinant (c) or wild type AcNPV (b). Proteins were recovered at 72 hr post-infection and an aliquot of each sample was resolved by gel electrophoresis. Uninfected cells were treated similarly (a). The resolved protein bands were either detected by staining with Coomassie brilliant blue (A), or blotted onto nitrocellulose membranes and detected immunologically with anti BTV-10 serum (B) as described

in Methods. The positions of NS1 and the AcNPV polyhedrin protein (P) are indicated. Molecular weights (in kD) are indicated on the left.

Fig. 5 Electron micrographs of NS1-derived tubules produced by *S. frugiperda* cells infected with recombinant viruses. *S. frugiperda* cells infected with AcNPV (panel a), or a recombinant virus (panels b-f) were fixed with 2% glutaraldehyde 72 hr post-infection and processed for electron microscopy as described in Methods. P, polyhedrin; V, virus particles; M, mitochondria; R, ribosomes; F, fibrous structure; T, tubules; N, nuclear membrane.

Fig. 6 Three-dimensional arrangement of the tubules in the infected *S. frugiperda* cells. (A) A series of representative virus (A-H) of the single arrays in a thin section of the tubules were photographed at 10° intervals of specimen tilt. (B) A schematic three-dimensional interpretation of the structure are represented (a, b and c).

Fig. 7 Reaction of bluetongue antibodies to recombinant baculovirus derived antigen using indirect ELISA: Recombinant AcBTV 10-6 infected cell extracts were adsorbed to the solid phase and examined using 1:100 to 1:12800 dilutions of sheep anti-BTV-2 serum (●--------●), or anti-BTV-10 serum (o------o), or anti-BTV-11 serum (▲———▲), or anti-BTV-13 serum (△———△), or anti-BTV-17 serum (□--------□), or with normal sheep serum (▨--------▨).

Fig. 8 SDS-PAGE of purified NS1 protein stained with Coomassie brilliant blue: AcBTV10-6 recombinant baculovirus infected cells were disrupted and tubules were recovered by sedimenting in sucrose gradient (10-50% w/v) centrifugation as described in Methods. The peak fractions containing NS1 were pooled and subjected to SDS-PAGE analysis. The proteins recovered from unpurified recombinant virus infected cells (1) are compared with NS1 protein purified through sucrose gradient centrifugation (2).

Table 1. *Amino acid composition of the predicted NS1 protein of BTV-10*

Amino acid (symbol)	No. of residues	% of Mol. Ratio
Alanine (A)	41	7.4
Arginine (R)	43	7.7
Aspartate (D)	32	5.7
Asparagine (N)	20	3.6
Cysteine (C)	16	2.9
Glutamate (E)	39	7.0
Glutamine (Q)	26	4.7
Glycine (G)	32	5.9
Histidine (H)	14	2.5
Isoleucine (I)	39	7.0
Leucine (L)	42	7.6
Lysine (K)	23	4.2
Methionine (M)	23	4.2
Phenylalanine (F)	26	4.7
Proline (P)	20	3.6
Serine (S)	25	4.5
Threonine (T)	23	4.2
Tryptophan (W)	13	2.3
Tyrosine (Y)	28	5.1
Valine (V)	27	4.9
Total amino acids	552	
Net charge	+2	
Size (daltons)	64,445	

CLAIMS

1. A process for producing a polypeptide comprising a bluetongue virus non-structural protein in antigenic form which comprises infecting susceptible insects or cultured insect cells with an expression vector having a DNA segment coding for said polypeptide.

2. A process according to Claim 1 wherein said DNA segment codes for bluetongue NS1 protein.

3. A process according to Claim 1 or Claim 2 wherein the polypeptide is produced in a morphological form resembling structures observed in bluetongue virus infected mammalian cells.

4. A process according to Claim 2 wherein said polypeptide is produced in the form of tubules.

5. A process according to any preceding claim wherein the expression vector comprises a recombinant baculovirus.

6. A recombinant baculovirus suitable for use in the process of any of Claims 1 to 5 comprising a DNA segment coding for a polypeptide comprising a bluetongue virus non-structural protein and a promoter capable of directing expression of said polypeptide in an infected insect or in infected insect cells.

7. A recombinant baculovirus as claimed in Claim 6 wherein the promoter is the polyhedrin promoter.

8. A polypeptide produced by the process of any of Claims 1 to 5.

9. Use of a polypeptide as claimed in Claim 8 to diagnose bluetongue virus infection in mammals.

10. Use of a polypeptide as claimed in Claim 8 in the manufacture of a vaccine.

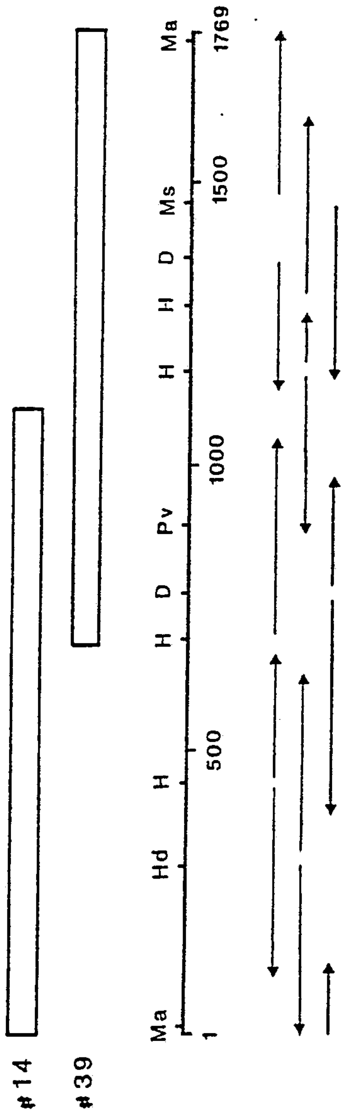
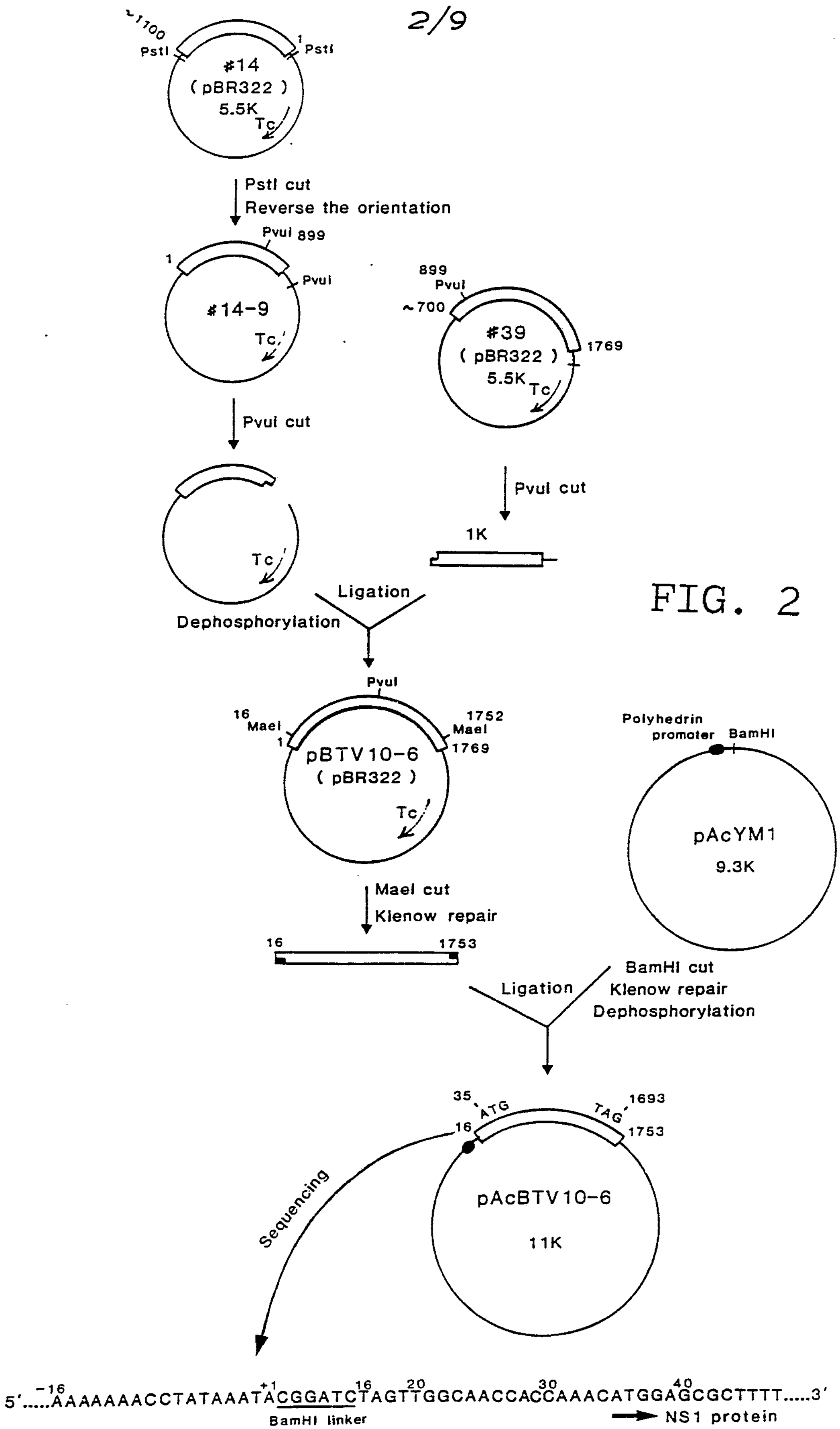


FIG. 1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PstI
#14
(pBR322)
5.5K
Tc
PstI cut
Reverse the orientation
PvuI 899
1
PvuI
#14-9
Tc
899
PvuI
~700
#39
(pBR322)
5.5K
Tc
1769
PvuI cut
PvuI cut
Tc
1K
Ligation
Dephosphorylation
PvuI
16
MaeI
1
1752
MaeI
1769
pBTV10-6
(pBR322)
Tc
Polyhedrin
promoter
BamHI
pAcYM1
9.3K
MaeI cut
Klenow repair
16
1753
Ligation
BamHI cut
Klenow repair
Dephosphorylation
35
ATG
16
TAG
1693
1753
pAcBTV10-6
11K
Sequencing
5'.....AAAAAAACCTATAAATACGGATCTAGTTGGCAACCACCAAACATGGAGCGCTTTT.....3'
−16
+1
16
20
30
40
BamHI linker
NS1 protein

FIG. 2

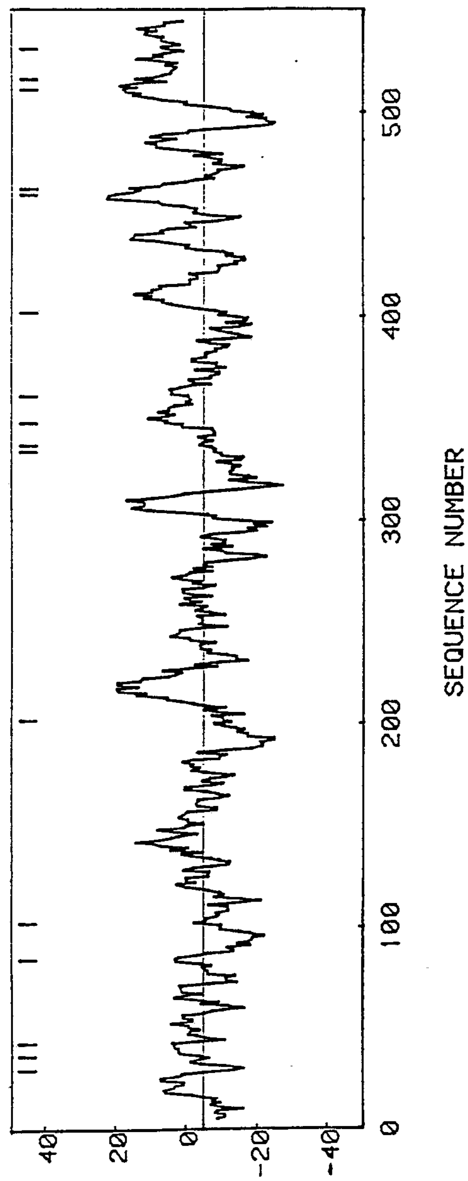


FIG. 3

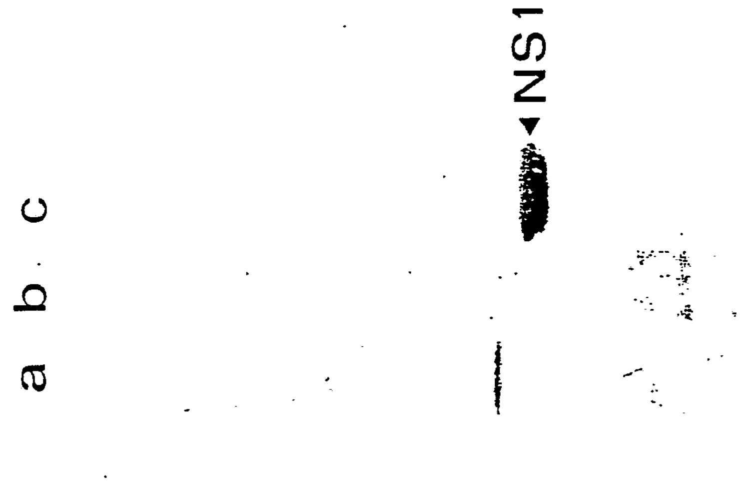


FIG. 4B

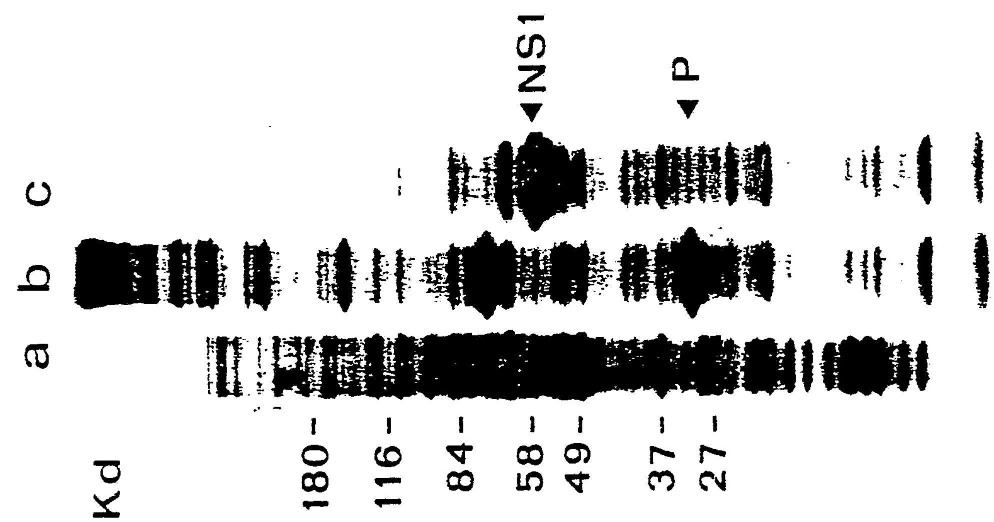


FIG. 4A

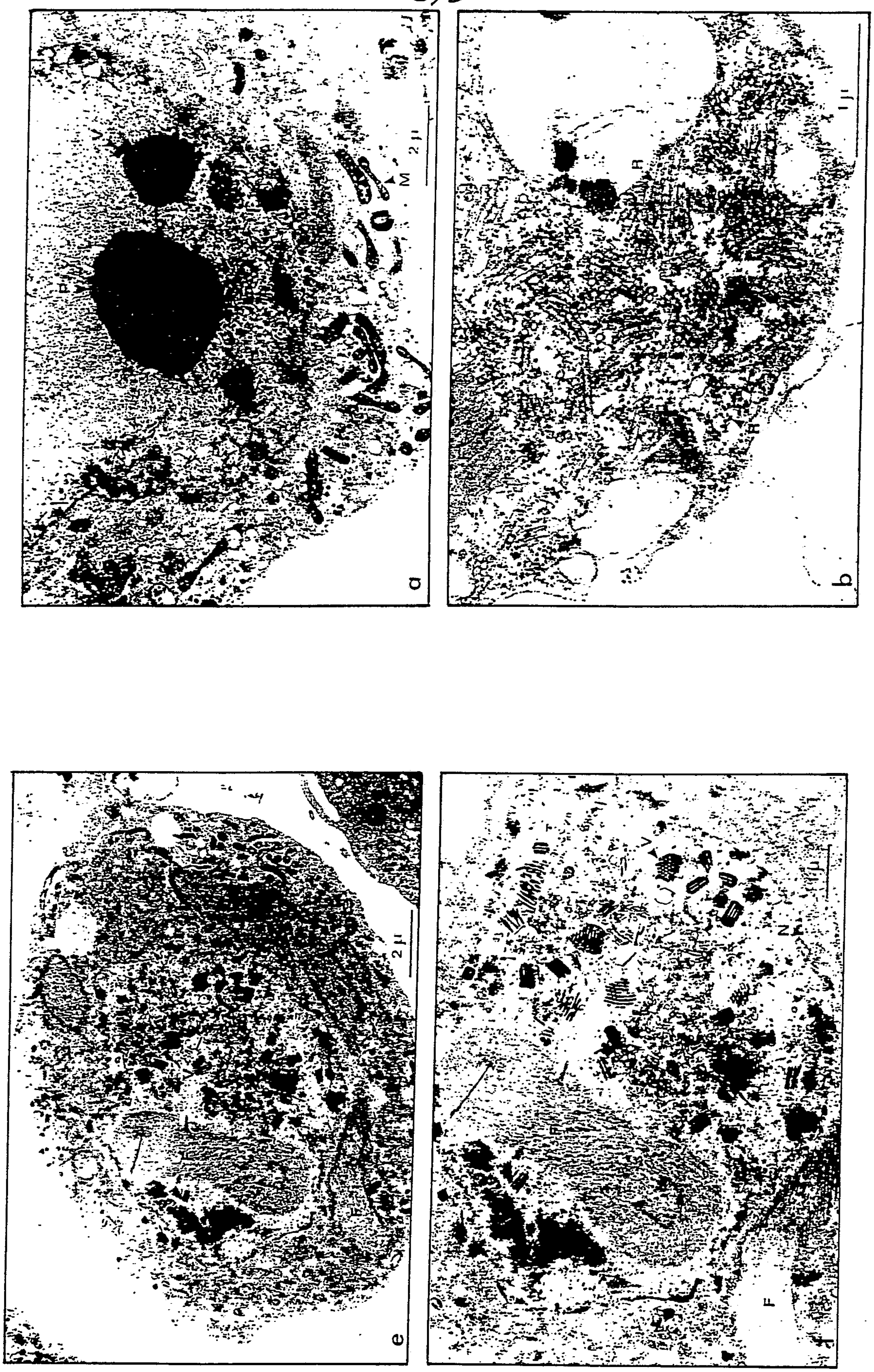


FIG. 5

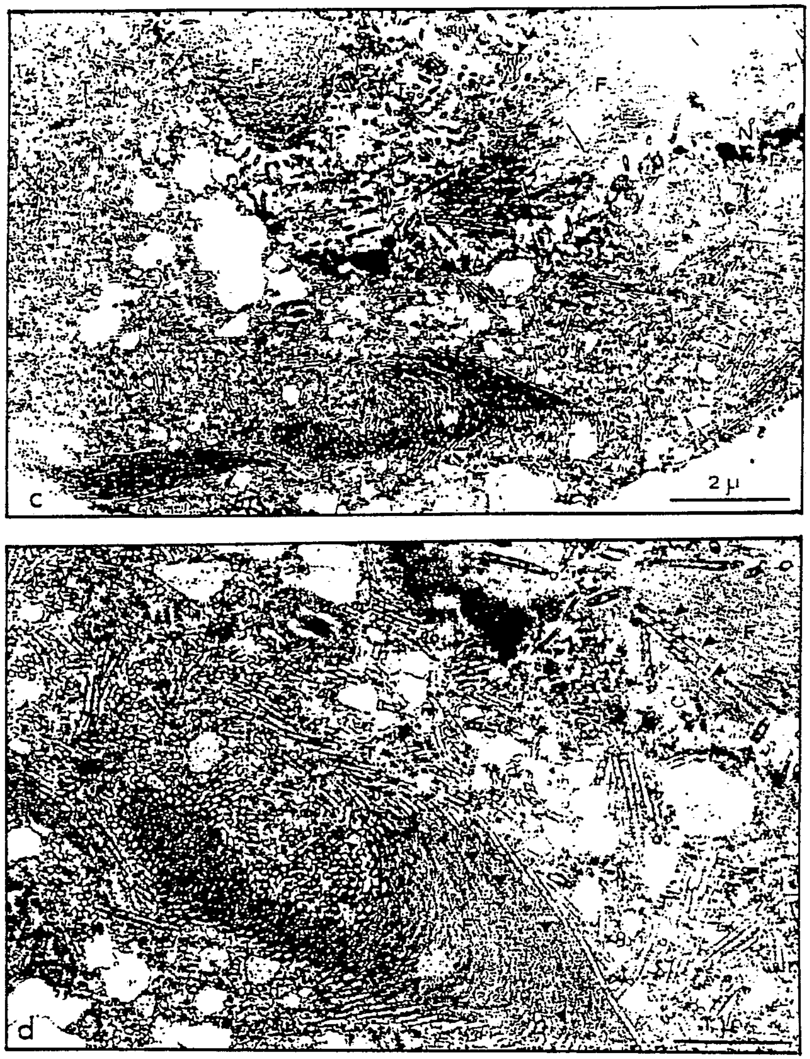


FIG. 5

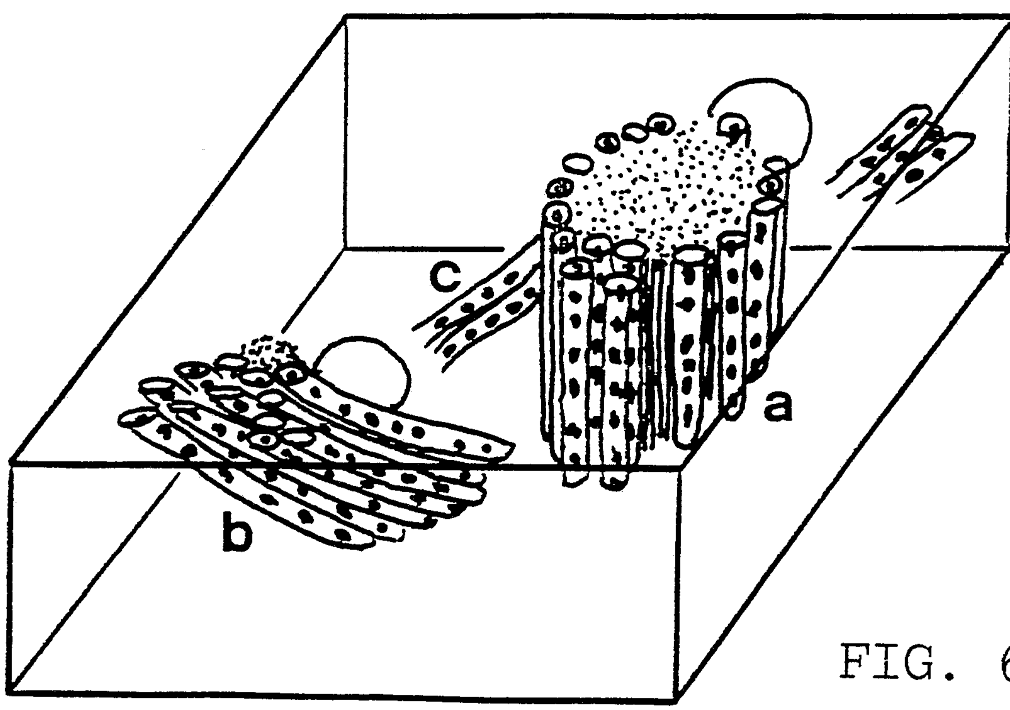
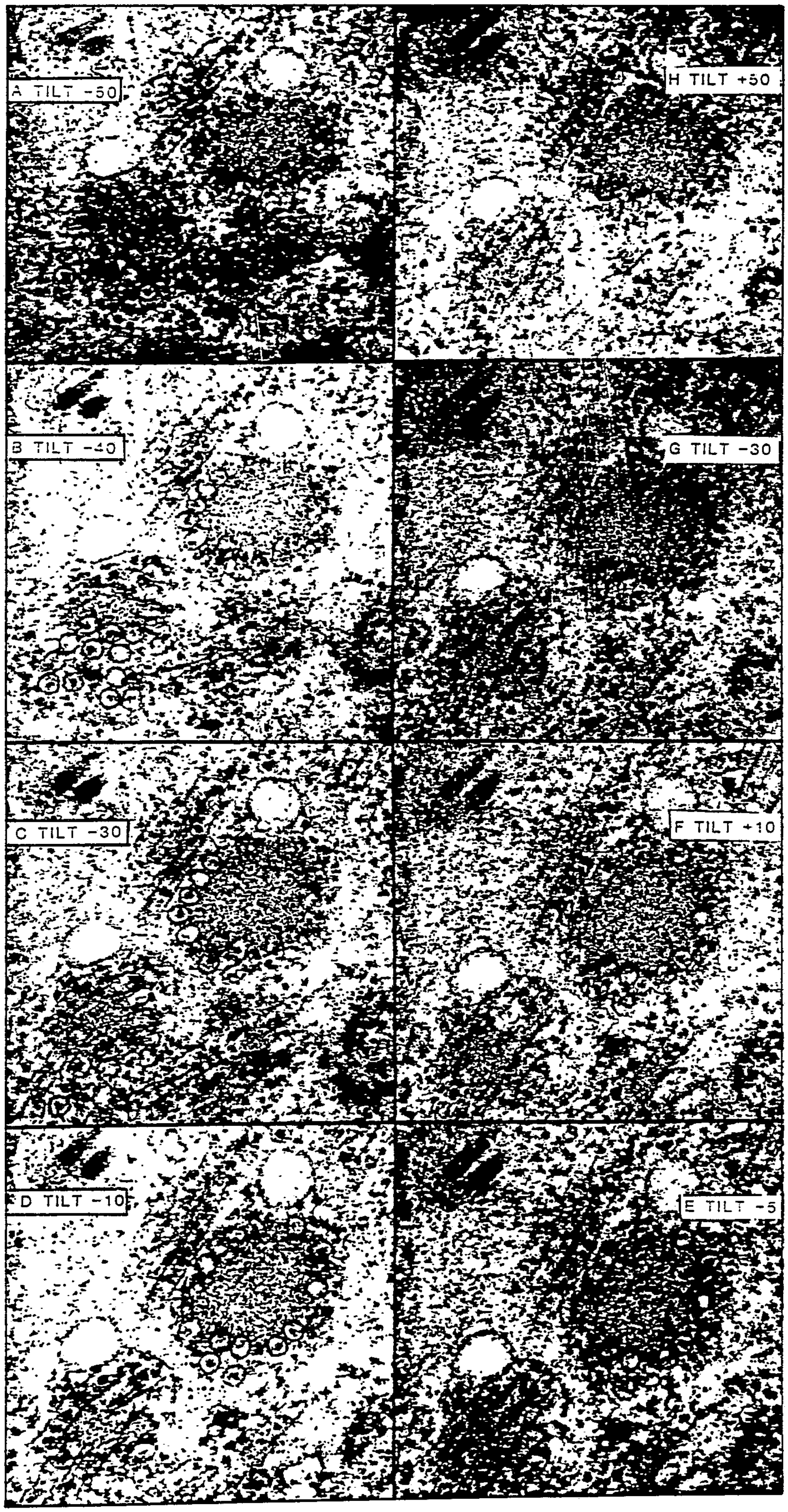


FIG. 6B

7/9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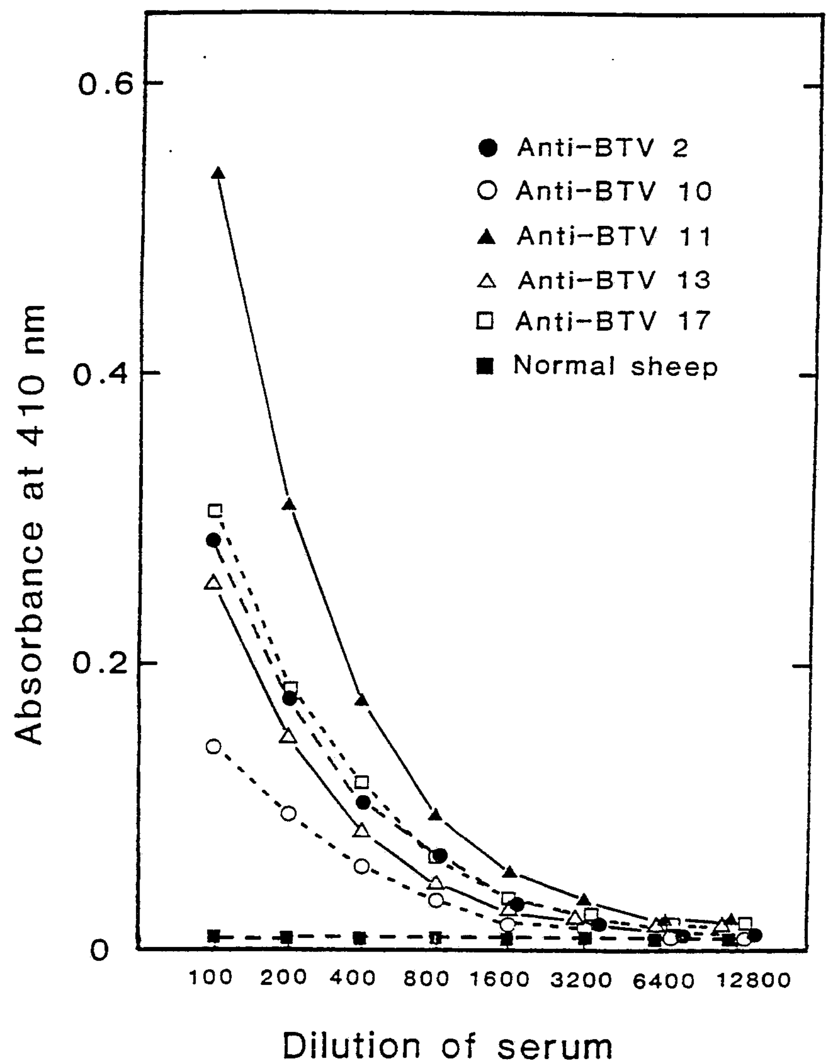
H TILT +50
B TILT -40
G TILT -30
C TILT -30
F TILT +10
D TILT -10
E TILT -5

FIG. 6A



0.6
0.4
0.2
0
Absorbance at 410 nm
Anti-BTV 2
Anti-BTV 10
Anti-BTV 11
Anti-BTV 13
Anti-BTV 17
Normal sheep
100
200
400
800
1600
3200
6400
12800
Dilution of serum

FIG. 7

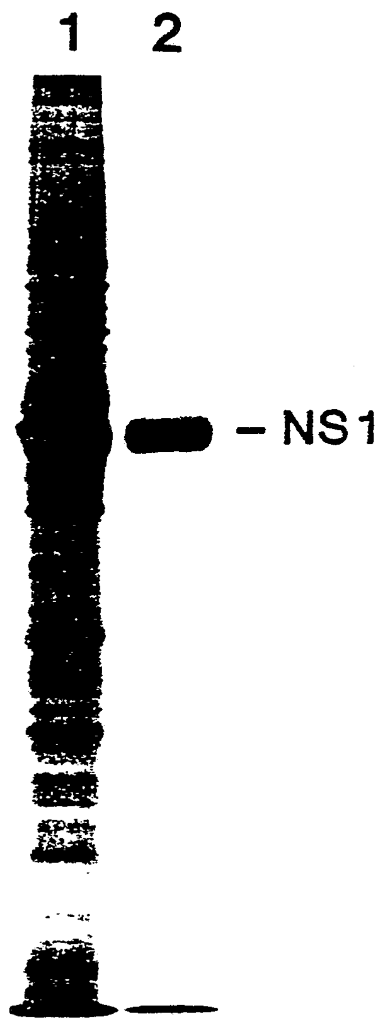


FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00939

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 15/46, A 61 K 39/15, C 12 N 15/86, G 01 N 33/569, //(C 12 N 15/00, C 12 R 1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Nucleic Acids Research, vol. 15, no. 17, 1987 IRL Press Ltd, Oxford, (GB) J. Lee et al.: "Complete sequence of the NS1 gene (M6 RNA) of bluetongue virus serotype 10", page 7207 see the whole article (cited in the application) --	1-9
Y	J. Gen. Virol., vol. 68, 1987, SGM (GB) S. Inumaru et al.: "Expression of bluetongue virus group-specific antigen VP3 in insect cells by a Baculovirus Vector: its use for the detection of bluetongue virus antibodies" page 1627 see the whole article (cited in the application) --	1-9

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th November 1989	12. 12. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, vol. 106, no. 21, 25 May 1987, (Columbus, Ohio, US), S. Inumaru et al.: "Production and characterization of the neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector", see page 549, abstract 174284t & Virology, 1987, 157(2), 472-9 (cited in the application) --	1-9
A	Biological Abstracts, vol. 84, 1987, (Philadelphia, PA, US) H. Huismans et al.: "A comparison of different cloned bluetongue virus genome segments as probes for the detection of virus-specified RNA" see abstract 52328 & Virology, 158(2): 373-380, 1987 (cited in the application) --	1-9
A	J. Gen. Virol. , vol. 67, 1986 SGM (GB) M.A.Purdy et al.: "Nucleotide sequence of cDNA clones encoding the outer capsid protein, VP5, of bluetongue virus serotype 10", pages 957-962 --	
A	EP, A, 0178435 (RESEARCH CORP.) 23 April 1986 --	
P,X	EP, A, 0279661 (OXFORD VIROLOGY LTD) 24 August 1988, see claim 1 --	1-9
P,X	Journal of Virology, vol. 62, November 1988 American Soc. for Microbiology (US) T. Urakawa et al.: "Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10" pages 3919-3927 see the whole article	1-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8900939

SA 30767

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 04/12/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0178435	23-04-86	AU-A- 4662885	06-03-86
		JP-A- 61158792	18-07-86
EP-A- 0279661	24-08-88	AU-A- 1195188	25-08-88

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82